Application for

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of

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and

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for

METHODS AND COMPOSITIONS FOR TRANSPOSITION USING MINIMAL SEGMENTS OF THE EUKARYOTIC TRANSFORMATION VECTOR *PIGGYBAC*

METHODS AND COMPOSITIONS FOR TRANSPOSITION USING MINIMAL SEGMENTS OF THE EUKARYOTIC TRANSFORMATION VECTOR *PIGGYBAC*

CROSS-REFERENCE TO RELATED APPLICATIONS

[01] This application makes reference to and claims priority to the following co-pending U.S. Patent Applications. The first application is U.S. Patent Application No. 10/001,189, entitled "Methods and Compositions for Transposition Using Minimal Segments of the Eukaryotic Transformation Vector *PiggyBac*," filed October 30, 2001, which claims priority to U.S. Provisional Patent Application No. 60/244,984, filed November 1, 2000, and U.S. Provisional Patent Application No. 60/244,667, filed on October 31, 2000. The second application is U.S. Provisional Patent Application No. _____, entitled "Methods and Compositions for Transposition Using Minimal Segments of the Eukaryotic Transformation Vector *PiggyBac*," filed April 15, 2004, Attorney Docket No. UNND-0061-4. The entire disclosure and contents of the four above-identified applications are hereby incorporated by reference.

GOVERNMENT INTEREST STATEMENT

[02] The United States Government has rights in this invention pursuant to USDA/NRI Grant 96-35302-3796, NIH-NIAID 1RO1AI40960, NIH/NIAID 1RO1AI48561, and NIH AI48561.

BACKGROUND

Field of the Invention

[03] The present invention relates generally to transposable elements, and more particularly to the transposon *piggyBac*.

Related Art

[04] Transposable elements (transposons) can move around a genome of a cell and are useful for inserting genes for the production of transgenic organisms. The *Lepidopteran* transposon *piggyBac* is capable of moving within the genomes of a wide variety of species,

and is gaining prominence as a useful gene transduction vector. The transposon structure includes a complex repeat configuration consisting of an internal repeat (IR), a spacer, and a terminal repeat (TR) at both ends, and a single open reading frame encoding a transposase.

[05] The *Lepidopteran* transposable element *piggyBac* was originally isolated from the TN-368 *Trichoplusia ni* cell culture as a gene disrupting insertion within spontaneous baculovirus plaque morphology mutants. *PiggyBac* is a 2475 bp short inverted repeat element that has an asymmetric terminal repeat structure with a 3-bp spacer between the 5' 13-bp TR (terminal repeat) and the 19-bp IR (internal repeat), and a 31-bp spacer between the 3' TR and IR. The single 2.1 kb open reading frame encodes a functional transposase (Cary *et al.*, 1989; Fraser *et al.*, 1983, 1995; Elick *et al.*, 1996a; Lobo *et al.*, 1999; Handler *et al.*, 1998).

[06] *PiggyBac* transposes via a unique cut-and-paste mechanism, inserting exclusively at 5' TTAA 3' target sites that are duplicated upon insertion, and excising precisely, leaving no footprint (Elick *et al.*, 1996b; Fraser *et al.*, 1996; Wang and Fraser 1993).

[07] Transient excision and interplasmid transposition assays have verified movement of this element in the SF21AE *Spodoptera frugiperda* cell line, and embryos of the *Lepidopteran Pectinophora glossypiella, Bombyx mori,* and *T.ni,* as well as the *Dipteran* species *Drosophila melanogaster, Aedes aegypti, Aedes triseriatus, Aedes albopictus, Anopheles stephensi* and *Anopheles gambiae.* There is also evidence of transposition in the Cos-7 primate cell line, and embryos of the zebra fish, *Danio rerio* (Fraser *et al.*, 1995; Buck *et al.*, 1996b; Fraser *et al.*, 1996; Elick *et al.*, 1997; Thibault *et al.*, 1999; Tamura *et al.*, 2000; Lobo *et al.*, 1999).

[08] The *piggyBac* element has been used successfully as a helper-dependent gene transfer vector in a wide variety of insect species, including the Mediterranean fruit fly, *C. capitata, D. melanogaster, Bombyx mori, P. glossypiella, Tribollium casteneum,* and *Ae. aegypti* (Handler *et al.*, 1998, 1999; Tamura *et al.*, 2000; Berghammer *et al.*, 1999).

[09] Excision assays using both wildtype and mutagenized *piggyBac* terminal sequences demonstrated that the element does not discriminate between proximal or distal duplicated ends, and suggest that the transposase does not first recognize an internal binding site and then scan towards the ends. In addition, mutagenesis of the terminal trinucleotides or the

terminal-proximate three bases of the TTAA target sequence eliminates excision at the altered terminus (Elick *et al.*, 1996b).

[10] Although the reported *piggyBac* vector is useful, length of genes that could be transferred is limited by the size of the other components of the vector. Minimizing the length of the vector to allow more room for the genetic material to be transferred would improve the versatility of the system and reduce costs of preparing synthetic vectors. Previously, the gene to be expressed or transduced was inserted into the middle of the *piggyBac* transposon in the plasmid p3E1.2. The final construct included the entire length of the *piggyBac* transposon (2475 bases) and flanking sequences derived from the baculovirus 25K gene region of approximately 813 bases, as well as the plasmid pUC backbone of 2686 bp, and an overall size of approximately 5962 bp. (In cloning sequences into the pUC vector, 12 bp of multiple cloning site DNA was lost). This size limited the effective size of genes that may be inserted, because plasmids larger than 10 KB are generally more difficult to construct, maintain, and transduce into host genomes.

[11] Another problem was that previous cloning regimens involved the excision of a gene, the promoter controlling the gene, and polyadenylation signals, from one plasmid followed by insertion into the *piggyBac* transfer vector. This procedure was often complicated by the lack of suitable restriction enzyme sites for these manipulations.

SUMMARY

[12] The present invention identifies the specific sequences in a mobile genetic element, the transposon *piggyBac*, and sequence configurations outside of *piggyBac*, that are minimally required for full functionality of the sequence as a transposon. Inserting DNA molecules into cells is enhanced using the methods and compositions of the present invention.

[13] The present invention solves problems in use of the *piggyBac* vector for gene transfer caused by lack of suitable restriction sites to cut the components needed for gene transfer, and limitations on the sizes (lengths) of genes transferred by use of this vector. Methods and compositions of the present invention enlarge the size of the gene that may be transferred in two ways. First, a minimal sequence cartridge may be easily amplified using primers

containing desired restriction endonuclease sites, and the cartridge may then be inserted into any plasmid containing the gene with its attendant promoter and polyadenylation signals intact, converting that plasmid into a *piggyBac* transposon. Second, a multiple cloning site may be inserted into a minimal plasmid vector, facilitating the insertion of genes in this more traditional plasmid vector. The vectors may both be used for applications including producing transgenic organisms, both plants and animals. The present invention has been successful in exemplary transpositions using the primate Cos-7 vertebrate cell line and embryos of the zebra fish, *Danio rerio*, among others.

[14] Methods and compositions are disclosed herein for transferring genes using the minimum internal and external sequences of the transformation vector *piggyBac*. In an embodiment of the invention, all non-essential sequences are removed, including the bulk of the *piggyBac* internal domain and the flanking baculovirus sequences. By means of the minimal *piggyBac* cartridge, a DNA molecule may be transferred from a plasmid into a host cell.

[15] According to a first broad aspect of the present invention, there is provided a DNA molecule comprising at least 163 consecutive nucleotide base pairs of the 3' terminal region beginning at the 3' terminal base pair, and at least 125 consecutive nucleotide base pairs of the 5' terminal region beginning at the 5' terminal base pair of the *piggyback* molecule, the region extending from the restriction site *Sac*I to the end of the *piggyback* molecule.

[16] According to a second broad aspect of the present invention, there is provided a genetic cartridge designated ITR.

[17] According to a third broad aspect of the present invention, there is provided a genetic cartridge designated ITR1.1k.

[18] According to a fourth broad aspect of the present invention, there is provided a vector designated pXL-Bac as shown in FIG. 3.

[19] According to a fifth broad aspect of the present invention, there is provided a vector designated pXL-BacII-ECFP as shown in FIG. 24.

[20] According to a sixth broad aspect of the present invention, there is provided a vector designated pBSII-ITR1.1k-ECFP as shown in FIG. 24.

BRIEF DESCRIPTION OF THE DRAWINGS

[21] The invention will be described in conjunction with the accompanying drawings, in which:

[22] FIG. 1 shows a p3E1.2 deletion series of plasmids and excision assay results; the p3E1.2 plasmid was used to make progressive deletions using the restriction endonuclease *Exo*III; three of the maximum deletion plasmids, p3E1.2-d-7, p3E1.2-d-8 and p3E1.2-d-9, were used to perform excision assays in *T.ni* embryos; p3E1.2d-7 and p3E1.2-d-8 plasmids retained the complete 3' terminal repeat configurations and were characterized by a similar excision frequency as the intact p3E1.2 plasmid; however, p3E1.2-d-9 did not yield any excision events, and sequencing results show that its 3' IR and part of the 31 bp spacer sequence are deleted;

FIG. 2 shows the pIAO-P/L insertion series of plasmids and presents interplasmid transposition assay results: (A) lists the pIAO-P/L series of plasmids' insertion sequences (SEQ ID NOS: 35-39) and their interplasmid transposition assay (IPTA) frequencies are shown; all the pIAO-P/L insertion plasmids were co-injected with the *piggyBac* helper plasmid, phspBac, and the target plasmid, pGDV1, into *T.ni* embryos to perform an interplasmid transposition assay; the results show that when the insertion sequence is less than 40 bp, the transposition frequency drops dramatically; (B) is a schematic representation of the pIAO-P/L series plasmids; the *piggyBac* sequence was PCR amplified from a p3E1.2B/X plasmid, polh*lacZ* is from a pD2/-gal *DraI*/*Nru*I fragment and AMP/ori was PCR amplified from a pUC18 plasmid; and (C1) is the nucleotide sequence of pIAO-P/L (SEQ ID NO: 57) and the amino acid sequences (SEQ ID NOS: 58-62) (C2) is the nucleotide sequence of pIAO-P/L-Lambda (2.2 kb) (SEQ ID NO: 63) and the amino acid sequences (SEQ ID NOS: 58-61 and 64-66);

FIG. 3 is a schematic representation of an ITR cartridge and pXL-Bac minimum *piggyBac* vectors; (A) the ITR cartridge may be amplified from the pIAO-P/L-589bp plasmid using an IR-specific primer; the amplified ITR may convert any existing plasmid into a *piggyBac* transposon, which may be mobilized if provided with the *piggyBac* transposase; (B) is a map of the pXL-Bac plasmid (MCS = multiple cloning site, *Bam*HI or *Bss*HII are restriction sites); (C1) the ITR cartridge nucleotide sequence (SEQ ID NO: 40); and (C2) is the nucleotide sequence (SEQ ID NO: 41) of pXL-Bac;

FIG. 4 is a restriction map of plasmid pCaSpeR-hs-orf (p32), containing a 2016 bp PCR *Bam*HI fragment containing *piggyBac* transposase and its terminator, cloned into *Bam*HI sites of pCaSpeR-hs;

FIG. 5(A) is a plasmid map showing the *piggyBac* ORF was amplified as a *Bam*HI cartridge from the p3E1.2 plasmid and cloned into pCaSpeR-hs plasmid, positioning it for transcriptional control by the hsp70 promoter; (B) is the nucleotide sequence (SEQ ID NO: 42) of pCaSpeR-hs-orf;

FIG. 6(A) is a plasmid map showing that the *piggyBac* ORF *Bam*HI cartridge from pCaSpeR-hs-orf was cloned into the pBSII (Stratagene) positioning it for transcription under control of the T7 promoter to form pBSII-IFP2orf; (B) is the nucleotide sequence (SEQ ID NO: 43) of pBSII-IFP2-orf;

FIG. 7(A) is a plasmid map showing that the *hsp70* promoter was excised from the pCaSpeR-hs plasmid by *Eco*R I and *Eco*R V digestion, followed by blunt ending, and cloned into pBSII-IFP2orf at the *Eco*R I and *Hin*d III (blunt ended) sites to form pBSII-hs-orf; (B) is a nucleotide sequence from pBSII-hs-orf;

FIG. 8(A) is a plasmid map showing that the IE1 promoter was PCR amplified from the pIE1FB plasmid (Jarvis *et al.*, 1990) and cloned into the pBSII-IFP2orf plasmid to form pBSII-IE1-orf; (B) is the nucleotide sequence (SEQ ID NO: 44) of pBSII-E1-orf;

FIG. 9(A) is a plasmid map showing that the base plasmid is pDsRed1-N1 (Clontech). The 3xP3 promoter was PCR amplified from pBac [3xP3-EYFPafm] (Horn and Wimmer, 2000) and cloned into the *Xho* I and *Eco*R I sites of pDsRed1-N1 to form the p3xP3-DsRed plasmid. The *piggyBac* ORF *Bam*HI cartridge from pCaSpeR-hs-orf was then cloned into the *Bgl*II site of p3xP3 DsRed positioning it under control of the CMV (cytomegalovirus) promoter to form p3xP3-DsRed-orf; (B) is the nucleotide sequence (SEQ ID NO: 45) of p3xP3-DsRed-orf. DsRed is a marker from Invitrogen and 3xP3 is a promoter specific for eyes of insects;

FIG. 10(A) is a plasmid map showing that the ITR cartridge was PCR amplified as a *Bam*HI fragment using a *piggyBac* internal repeat specific primer (5'-GGATCCCATGCGTCAATTTTACGCA-3') (SEQ ID NO: 1) and pIAO-P/L-589bp plasmid as a template, and cloned into the pCRII plasmid (Invitrogen) to form the pCRII-ITR

plasmid; (B) is the nucleotide sequence of pCRII-ITR (SEQ ID NO: 46) and the amino acid sequence (SEQ ID NO: 47);

FIG. 11 is a plasmid map showing that the ITR *Bam*HI cartridge was recovered from the pCRII-ITR plasmid and religated, then cut with *Bss*HII and cloned into the *Bss*HII sites of the pBSII plasmid (Stratagene) to form pBS-ITR(rev) plasmid. The Multiple Cloning Sites were PCR amplified as a *Bgl*II fragment from the pBSII plasmid and were cloned into the *Bam*HI site to the pXL-Bac plasmid;

FIG. 12(A) is a plasmid map showing that the P element enhancer trap plasmid pP {PZ} (from Dr. O'Tousa, Univ. of Notre Dame) was digested with *Hin*d III then self-ligated to produce the p(PZ)-*Hin*dIII plasmid. The ITR cartridge was excised using *Sal* I and *Not* I (blunt-ended) from pCRII-ITR and then cloned into the blunt ended *Hin*d III site to form p(PZ)-Bac. The 3xP3-EYFP was PCR amplified as an *Spe* I fragment from pBac[3xP3-EYFPafm] (Horn and Wimmer, 2000) and cloned into the *Spe* I site of p(PZ)-Bac plasmid to form the p(PZ)-Bac-EYFP plasmid; (B) is the nucleotide sequence (SEQ ID NO: 48) of p(PZ)-Bac-EYFP;

FIG. 13(A) is a plasmid map showing that the P element enhancer trap plasmid pP{PZ} (from Dr. O'Tousa, Univ. of Notre Dame) was digested with *Hin*dIII then self-ligated to produce the p(PZ-)-*Hin*dIII plasmid. The ITR cartridge was excised using *Sal* I and *Not* I (blunt ended) from pCRII-ITR and then cloned into the blunt ended *Hin*d III site to form p(PZ)-Bac. The 3xP3-ECFP was PCR amplified as an *Spe* I fragment from pBac[3xP3-ECFPafm] (Horn and Wimmer, 2000) and cloned into the *Spe* I site of the p(PZ)-Bac plasmid to form the p(PZ)-Bac-ECFP plasmid; (B) is the nucleotide sequence (SEQ ID NO: 49) of p(PZ)-Bac-ECFP;

FIG. 14(A) is a plasmid map showing that the P element enhancer trap plasmid pP{PZ} (from Dr. O'Tousa, Univ. of Notre Dame) was digested with *Hin*d III then self-ligated to produce the p(PZ)-*Hin*dIII plasmid. The ITR cartridge was excised using *Sal* I and *Not* I (blunt ended) from pCRII-ITR and then cloned into the blunt ended *Hin*dIII site to form p(PZ)-Bac. The 3xP3-EGFP was PCR amplified as an *Spe* I fragment from pBac[3xP3-EGFPafm] (Horn and Wimmer, 2000) and cloned into the *Spe* I site of the p(PZ)-Bac plasmid to form the p(PZ)-Bac-EGFP plasmid; (B) is the nucleotide sequence (SEQ ID NO: 50) of p(PZ)-Bac-EGFP;

FIG. 15(A) is a plasmid map showing that the 3xP3-EYFP gene was PCR amplified as an *Spe* I fragment from pBac [3xP3-EYFPafm] (Horn and Wimmer, 2000) and cloned into the *Spe* I site of the pXL-Bac plasmid to form the pXL-Bac-EYFP plasmid; (B) is the nucleotide sequence (SEQ ID NO: 51) of pXL-Bac-EYFP;

FIG. 16(A) is a plasmid map showing that the 3xP3-EGFP gene was PCR amplified as an *Spe* I fragment from pBac [3xP3-EGFPafm] (Horn and Wimmer, 2000) and cloned into the *Spe* I site of the pXL-Bac plasmid to form the pXL-Bac-EGFP plasmid; (B) is the nucleotide sequence (SEQ ID NO: 52) of pXL-Bac-FGFP;

FIG. 17(A) is a plasmid map showing that the 3xP3-ECFP gene was PCR amplified as an *Spe* I fragment from pBac [3xP3-ECFPafm] (Horn and Wimmer, 2000) and cloned into the *Spe* I site of the pXL-Bac plasmid to form the pXL-Bac-ECFP plasmid; (B) is the nucleotide sequence (SEQ ID NO: 53) of pXL-Bac-ECFP;

FIG. 18(A) is a plasmid map showing that the 3xP3-ECFP was PCR amplified as an *Spe* I fragment from pBac[3xP3-ECFPafm] (Horn and Wimmer, 2000) and cloned into the *Spe* I site of the pBS-ITR plasmid to form the pBS-ITR-ECFP plasmid; (B) is the nucleotide sequence (SEQ ID NO: 54) of pBS-ITR-ECFP;

FIG. 19(A) is a plasmid map showing that the 3xP3-EGFP was PCR amplified as an *Spe* I fragment from pBac[3xP3-EGFPafm] (Horn and Wimmer, 2000) and cloned into the *Spe* I site of the pBS-ITR plasmid to form the pBS-ITR-EGFP plasmid; (B) is the nucleotide sequence (SEQ ID NO: 55) of pBS-ITR-EGFP;

FIG. 20(A) is a plasmid map showing that the 3xP3-EYFP was PCR amplified as an *Spe* I fragment from pBac[3xP3-EYFPafm] (Horn and Wimmer, 2000) and cloned into the *Spe* I site of the pBS-ITR plasmid to form the pBS-ITR-EYFP plasmid; (B) is the nucleotide sequence (SEQ ID NO: 56) of pBS-ITR-EYFP;

FIG. 21(A) is a plasmid map showing that the Actin 5c promoter was cloned as a *Bam*HI and *Eco* I fragment (bases 3046 to 3055 of SEQ ID NO: 67) from the pHAct5cEFGP plasmid (from Dr. Atkinson, UC Riverside) into the *Bam*HI and *Eco*RI sites of the pBSII plasmid (Stratagene) to form the pBSII-Act5c plasmid. The *piggyBac* ORF *Bam*HI cartridge from pCaSpeR-hs-orf was then cloned into pBSII-Act5c plasmid under control of the Act5c promoter; (B) is the nucleotide sequence (SEQ ID NO: 67) of pBSII-Act5c-orf;

FIG. 22 is the nucleotide sequence (SEQ ID NO: 68) of pCaSpeR-hs-pBac;

FIG. 23 is a comparison of natural and optimized *piggyBac* nucleotide sequences (SEQ ID NOS: 69 and 70) wherein "optimizing" means using codons specific for insects;

FIG. 24 (A) shows a plasmid construction map of pCaSpeR-hs-orf. The *piggyBac* ORF *Bam*HI cassette was cloned as a PCR product into the *Bam*HI site of the pCaSpeR-hs vector for expression using the *hsp70* promoter. (B) shows a plasmid construction map of p(PZ)-Bac-EYFP. The 7kb p(PZ) *Hin*d III fragment containing *LacZ*, *hsp70* and Kan/ori sequences was recircularized to form the p(PZ)-7kb plasmid. The ITR cartridge (Li *et al.*, 2001) was digested with *Not* I and *Sal* I, blunt ended, and inserted into the blunt ended *Hin*d III site of the p(PZ)-7kb plasmid. A 3xP3-EYFP (Horn and Wimmer, 2000) *Spe* I fragment was then inserted into the *Xba* I site to form p(PZ)-Bac-EYFP. (C) is an illustration of the pBSII-ITR1.1k-ECFP minimal *piggyBac* vector, which contains a minimal *piggyBac* cartridge with the terminal and subterminal inverted repeats facing each other, and tagged with a 3xP3-ECFP marker. (D) is an illustration of the more traditional *piggyBac* minimal vector pXL-BacII-ECFP plasmid;

[23] FIG. 25 is a schematic illustration of *piggyBac* internal deletion series plasmids based on the pIAO-P/L-589bp. The 5' Terminal Repeat is 35 bp in length and the 3' Terminal Repeat is 63 bp in length. The p(PZ)-Bac-EYFP plasmid contains only the *piggyBac* terminal repeat regions using the ITR cartridge of Li *et al.*, 2001;

[24] FIG. 26 (A) shows direct PCR analysis of transformed flies. A total of three sets of PCRs were used to verify the *piggyBac* insertion. The first set (IFP2_R1 + MF34 primers) detects the 5' terminal region (115 bp), the second set (IFP2_L + MF34 primers) detects the 3' terminal region (240 bp), and the third set (IFP2_R1 + IFP2_L primers) detects the presence of the external spacer sequence (945 bp). (B) shows the PCR results in which (a) all transformed strains have the correct sized fragment confirming the 5' terminal region, and there is also a weak band evident in the w1118 strain, (b) all transformed strains have the correct sized fragment confirming the 3' terminal region, and this fragment is absent in the w1118 strain, and (c) no external spacer sequence fragment is evident in any of the transformed strains;

[25] FIG. 27 shows southern hybridization analysis of internal deletion plasmid transformed strains. Genomic DNAs from selected strains and the pBSII-ITR1.1k-ECFP

plasmid control were digested with *Hind* III and hybridized to the pBSII-ITR1.1k-ECFP plasmid probe. The 2.96 kb pBSII and 1.16 kb ECFP marker should be present in all strains. (A) shows a map of the pBSII-ITR1.1k-ECFP plasmid showing the size of expected fragments. (B) shows that all transformed strains exhibit the two diagnostic bands (2.96 kb and 1.16 kb) and at least two additional bands reflecting the *piggyBac* terminal adjacent sequences at the site of integration. A weak 1.3 kb band is also observed in all strains. The reason that the two additional bands are much weaker than the diagnostic bands may be that these two additional bands represent the *piggyBac* termini containing bands, which contain only 200~300 bp of AT rich sequences that will be hybridized by the probe. The normal 60°C washes may wash away these weak hybridizations, thus causing the weak band on the blot; and

[26] FIG. 28 shows a schematic illustration of the locations of the two short repeat sequences in *piggyBac*. The repeats with the locations in bold are within the region between R and R1, or L and L2, which appear to be the important regions based on the transformation results discussed in the present invention. These repeats may also be found in some other position of the *piggyBac* sequence. From the present invention, it appears that a minimum of one set of these repeats on either side of the internal domains are useful for the transposon to permit full transforming capability.

DETAILED DESCRIPTION

[27] It is advantageous to define several terms before describing the invention. It should be appreciated that the following definitions are used throughout this application.

Definitions

[28] Where the definition of terms departs from the commonly used meaning of the term, applicant intends to utilize the definitions provided below, unless specifically indicated.

[29] For the purposes of the present invention, the term "spacer" refers to sequences, for example from about 3 bp to about 31 bp or more in length, separating the 5' and 3' (respectively) terminal repeat and internal repeat sequences of the *piggyBac* transposon.

[30] For the purposes of the present invention, the term "vector" refers to any plasmid containing *piggyBac* ends that is capable of moving foreign sequences into the genomes of a target organism or cell.

[31] For the purposes of the present invention, the term "plasmid" refers to any self-replicating extrachromosomal circular DNA molecule capable of maintaining itself in bacteria.

[32] For the purposes of the present invention, the term "transgenic organism" refers to an organism that has been altered by the addition of foreign DNA sequences to its genome.

[33] For the purposes of the present invention, the term "genetic construct" refers to any artificially assembled combination of DNA sequences.

[34] For the purposes of the present invention, the term "helper construct" refers to any plasmid construction that generates the *piggyBac* transposase gene product upon transfection of cells or injection of embryos.

Description

[35] The minimal sequence cartridges of the present invention facilitate transposition of DNA molecules of interest into cells, and production of transgenic organisms that include the transferred DNA molecule in some or all of their cells. A DNA molecule(s) is excised from a genetic (transformation) construct, and is transferred to a cell where it is inserted into the cell's genome. The DNA molecule is accompanied by regulatory elements sufficient to allow its expression in the host cell. "Cell" as used herein includes eukaryotic and prokaryotic cells. The genetic transposition construct includes a DNA molecule to be transferred flanked by a pair of transposon terminal inverted repeat nucleotide sequences from the *piggyBac* transposon. The DNA molecule to be transferred may be any molecule capable of being expressed in a host cell and/or transgenic organism. The method would also transfer cells not able to be expressed.

[36] In the present invention, excision (Elick *et al.*, 1996b) and interplasmid transposition assays (Lobo *et al.*, 1999) were used to determine the relative importance of sequences internal to, or external to, the terminal repeat (TR) and internal repeat (IR) sequence configurations for movement of the *piggyBac* element.

[37] It was found that progressive deletions within the internal sequence of the element have no noticeable effect on either excision or transposition capabilities. In contrast, deletion of the 3' IR eliminated excision of the element. Construction of vectors having only intact 5' and 3' repeat domains regenerates mobility of the plasmids when supplied with a helper vector expressing a transposase. These features permitted construction of a set of minimal vectors for use in transformation experiments.

[38] The length of the intervening sequence between *piggyBac* termini in the donor plasmid also affects the *piggyBac* transposition frequency. In an embodiment of the present invention, a minimal distance of 55 nucleotide base pairs (bp) may be used between target sites and termini to provide for movement of the element. This suggests that the *piggyBac* transposase binds the termini simultaneously before any cleavage may occur, and/or that the formation of the transposition complex requires DNA bending between the two termini.

[39] An aspect of this invention is that it allows the design of minimally sized genetic vectors that are functional for efficient insertion of genes into host genomes, in particular animal, plant, and insect genomes.

[40] Useful plasmids created are:

A) **A Transposition *PiggyBac* ITR Cartridge Plasmid**: PCR amplifications and restriction endonuclease cleavage and ligation allowed insertion of a 702bp fragment containing sequences for *piggyBac* mobility into any given plasmid of choice, converting the recipient plasmid into an operational transposable sequence capable of being mobilized into an animal genome using the *piggyBac* transposase gene or purified protein. The pCRII (Invitrogen) plasmid re-amplification using specified primers allows this ITR cartridge to be inserted into any plasmid.

B) **Operational Transposable Vectors (pXO and pXL-Bac)**: Standard restriction endonuclease cleavage and ligation allows insertion of any gene of choice between the minimal sequences of the *piggyBac* transposon necessary for transposition into the genome of an animal. The total size of the resulting plasmid is preferably not larger than 10kb.

[41] According to an embodiment of the present invention, the inverted repeat configuration indicated as [TTAA/TR/IR . . IR/3lbp/TR/TTAA] may be utilized to obtain a *piggyBac* transposon. This observation was arrived at through structured deletion

mutagenesis within the *piggyBac* transposon sequence and examining the properties of both excision and interplasmid transposition of the deleted product.

[42] Additionally, according to an embodiment of the present invention, an insertion sequence between the target site on a plasmid having the terminal repeat configuration [IR/3lbp/TR/TTAA...insertion sequence...TTAA/TR/IR] may be approximately 55bp to achieve mobility.

[43] For ease of manipulation, a cartridge having the configuration [IR/3lbp/TR/TTAA... .589...TTAA/TR/IR] which may be inserted within a plasmid, converting that plasmid into a functional *piggyBac* transposon, was constructed. The cartridge was cloned into the plasmid pCRII (Invitrogen). A cartridge is defined herein as a nucleic acid molecule of a specified construction (plasmid) that may be inserted into a vector.

[44] A cartridge was derived from circularization of the construct A and cutting the construct A with *Bss*HII to cleave at a unique *Bss*HII site within the 589bp spacer. This yielded a fragment *Bss*HII...TTAA/TR/3lb/IR/BamHI/IR/TR/TTAA...*Bss*HII. Construct B was derived from a pBSII (Stratagene) plasmid by *Bss*HII deletion of the multiple cloning site (MCS). The linearized fragment was then inserted into the pBSII^{a}*Bss*HII backbone. An MCS primer was synthesized and inserted in the *Bam*HI site.

[45] Construct A allows ease of construction of genetic vectors through use of a simple 702bp cartridge that may be inserted into any existing plasmid to convert it immediately into a functional transposon.

[46] Construct B allows ease of insertion of any genetic sequence into a plasmid having the minimal terminal sequence requirement for *piggyBac* mobility. The advantage of this construct is it provides a minimal backbone cloning vector for *piggyBac* transposon construction.

[47] A kit is contemplated that would contain the two vector constructs along with the original p3E1.2, and/or a helper construct allowing constitutive production of *piggyBac* transposase in virtually any animal system. Promoter driven expression of the *piggyBac* transposase using either RSV LTR sequences CMV early promoter, AcMNPV/IE-1 promoter of poly-ubiquitin promoter, among others, is also contemplated.

[48] Excision assays of plasmids containing progressive deletions of the *piggyBac* internal sequence revealed that the 5', and 3' IR, spacer, and TR configurations are sufficient for *piggyBac* movement when provided with a transposase in the trans position. Interplasmid transposition assays of plasmids having different sequence lengths between the target sites demonstrated a minimal 55 bp intervening sequence provides for satisfactory *piggyBac* transposition, whereas lengths less than 40 bp result in dramatic decreases in frequency of transpositions. These results suggest that the *piggyBac* transposase binds the termini simultaneously before cleavage, and/or that the formation of the transposition complex requires DNA bending between the two termini. Based on these results, a 702 bp cartridge having a minimum *piggyBac* 5' and 3' terminal region configuration and intervening sequence was constructed. The ability of this region to convert any existing plasmid into a non-autonomous *piggyBac* transposon was verified. A minimal *piggyBac* vector, pXL-Bac, that contains an internal multiple cloning site sequence between the terminal regions, was also constructed. These vectors facilitate manipulations of the *piggyBac* transposon for use in a wide variety of hosts.

[49] The excision assay provides a rapid way to characterize essential sequences involved in *piggyBac* transposition. The p3E1.2-d-7 and p3E1.2-d-8 plasmids, which retain the entire 3' and 5' IR, spacer and TR sequences, exhibit precise excision. In contrast, the p3E1.2-d-9 plasmid that retains the entire 5' terminal region and only 36 bp of the 3' terminal domain, including the TR and a portion of the 31 bp spacer, does not excise at a detectable frequency. The requirement for an internal 3' IR sequence in the excision process suggests that the IR region might play an essential role in transposase recognition or cleavage of the target site.

[50] An alternative explanation is that simply shortening the internal sequence may hinder the formation of a transposition complex, or the binding of transposase to two termini simultaneously. A similar result is observed with the IS5O elements for which the lengthening of Tn5 internal sequences increases the transposition frequency (Goryshin *et al.*, 1994). However, insertion of a KOα fragment into the p3E1.2-d-9 at the *Sph*I site did not improve the frequency of precise excision events recovered in the excision assay, suggesting that the length of the internal domain is less important than the presence of an intact IR sequence in excision of the *piggyBac* element.

[51] The interplasmid transposition assays of pIAO-P/L series plasmids demonstrate that when the external sequence separating the terminal repeats is at least 55 bp, the transposition

frequency is over 10^{-4}, while reducing the length to less than 40 bp depresses the frequency of transposition. The inhibition of *piggyBac* transposition as terminal sequences are brought closer together, suggests that formation of a transposition complex likely precedes DNA cleavage or nicking, and the shorter distances between these termini do not allow proper bending of the sequences to permit formation of the complex, or result in steric hindrance of transposase binding at the termini.

[52] These results also imply a necessity for transposase binding of both termini simultaneously before any cleavage (or nicking) may occur. If the simultaneous binding were not necessary, then the transposase could bind one terminal repeat, cleave it, and then bind the second to cleave, and transposition should occur with equivalent frequencies even with smaller intervening sequences.

[53] Interplasmid transposition assays using pCRII-ITR (FIG. 10) verify that the terminal configuration IR, spacer, TR are the minimum sequence requirements for efficient *piggyBac* transposition. The rest of the *piggyBac* internal sequence is not required if transposase is provided in trans configuration. With the ITR fragment, a minimum *piggyBac* vector may easily be constructed from any plasmid which reduces vector size and leaves maximum space for desired foreign genes.

[54] Inserting the ITR fragment into pBlueScript II (Stratagene), converts the plasmid into a transposable element that moves with a frequency similar to the intact *piggyBac* element. This ITR cartridge facilitates the construction of *piggyBac* transformation vectors from existing plasmids. In addition, the co-integration of the Amp/ori sequences from the donor plasmid into the genome provides an easy way to locate the insertion site because these insertions may be recovered by restriction enzyme digestion, religation, and transformation. The pXL-Bac (FIG. 11) minimum *piggyBac* vector replaces the internal sequence of the *piggyBac* transposon with a multiple cloning site. This plasmid allows any desired foreign genes or sequences to be easily inserted between *piggyBac* termini for movement in the presence of a helper plasmid. These constructs provide useful tools for the examination and use of *piggyBac* as a gene transfer vector in a wide variety of organisms.

EXAMPLES

Example 1: Excision Assay of p3E1.2 Internal Deletion Series in *T.ni*

[55] The analysis was begun using three plasmids having the most extensive internal deletions, p3E1.2-d-9, p3E1.2-d-8 and p3E1.2-d-7. Sequencing of these three plasmids revealed that p3E1.2-d-8 and p3E1.2-d-7 retained 163 bp and 303 bp of the 3' terminal region, respectively, including the IR, 31bp spacer, and TR sequence. The p3E1.2-d-9 deletion plasmid retained only 36 bp of the 3' terminal domain, including the 3' TTAA target site, 3' TR and a portion of the 31 bp spacer, but lacked the 3' IR sequence.

[56] Embryos of *T.ni* were injected with combinations of each of the p3E1.2 deletion plasmids and the phspBac helper plasmid. Loss of *piggyBac* sequences from the deletion series plasmids renders the plasmids resistant to *Bsi*WI and *Sph*I digestion. Transformation of Hirt extract DNAs digested with *Bsi*WI and *Sph*I were compared with transformations employing equal amounts of uncut DNA as a control to determine the frequency of excision. Precise excision events were initially identified by a quick size screen for the characteristic 3.5 kb plasmid in recovered colonies, and these plasmids were then sequenced to confirm the precise excision events.

[57] A quick size screen method is used to quickly identify the plasmids with changed size directly from colonies (Sekar, 1987). Colonies at least 1 mm in diameter are picked up with pipette tips and resuspended in 10 ml protoplasting buffer (30 mM Tris-HCl pH 8.0, 50 mM NaCl, 20% Sucrose 5 mM EDTA, 100 mg/ml RNase, 100 mg/ml Lysozyme) in the Lux 60 well mini culture plate. A 0.9% agarose gel containing ethidium bromide is preloaded with 4.5 ml lysis solution (80 mM Tris, 0.5% Sucrose, 0.04% Bromophenol Blue, 2% SDS, 2.5 mM EDTA) per well. The bacterial suspension is then loaded into the wells and the gel electrophoresed. Two kinds of markers are needed to distinguish the plasmids with changed size. One is the colony from the control plate or the original plasmid, another is a molecular weight marker. The plasmids with a difference of 500 bp or greater in size are easily distinguished. Both the p3E1.2-d-8 and p3E1.2-d-7 yielded precise excision events at about the same relative frequency, while no excision events were recovered with the maximum deletion plasmid p3E1.2-d-9 (FIG. 1).

Example 2: Minimal Distance Required between Termini for Movement of a *PiggyBac* Transposon Construct

[58] The interplasmid transposition assay was carried out essentially as previously described by Lobo *et al.* (1999), Thibault *et al.* (1999) and Sarkar *et al.* (1997a). Embryos were injected with a combination of 3 plasmids. The donor plasmid, pB(KOα), carried a *piggyBac* element marked with the kanamycin resistance gene, ColE1 origin of replication, and the *lacZ* gene. The transposase providing helper plasmid, pCaSpeR-pB-orf, expressed the full length of the *piggyBac* ORF under the control of the *D. melanogaster hsp70* promoter. The target *B. subtilis* plasmid, pGDV1, is incapable of replication in *E. coli*, and contains the chloramphenicol resistance gene. Upon transposition of the genetically tagged *piggyBac* element from pB(KOα) into the target plasmid pGDV1 with the help of the transposase provided by the helper pCaSpeR-pB-orf that expresses the *piggyBac* transposase protein from a minimal *hsp70* promoter (see FIG. 4), only the interplasmid transposition product would be able to replicate in *E. coli* and produce blue colonies on LB/kan/cam/X-gal plates. Embryos were injected with a mixture of the transposase-providing helper plasmid, phspBac, one of the pIAO-P/L series plasmids as the donor, and the pGDV1 target plasmid. Transposition of the tagged *piggyBac* element from any of the pIAO- P/L plasmids into the target plasmid pGDV1 allows the recipient pGDV1 to replicate in *E. coli* and produces blue colonies on LB/Amp/Cam/X-gal plates.

[59] A total of 10 blue colonies were randomly picked from each transformation and prepared for sequencing analysis. Initial sequence analysis of the terminal repeat junction showed that all of the sequenced clones had the distinctive duplication of a TTAA tetranucleotide target site, a characteristic feature of *piggyBac* transposition. A random set of those clones for which the 5' terminus had been sequenced were also examined at their 3' terminus to confirm the duplication of the TTAA site at both ends. The accumulated results confirmed transposon insertion at 12 of the 21 possible TTAA target sites in the pGDV1 plasmid, all of which were previously identified as insertion sites in *Lepidopteran* assays by Lobo *et al.* (1999) and Thibault *et al.* (1999).

[60] The relative frequency at which a given pIAO-P/L series plasmid was able to undergo transposition into the target plasmid correlated with the sizes of the intervening sequence between the termini. With intervening sequences greater than 55 bp, the transposition frequency was over 1.2×10^{-4}, which is consistent with the frequency obtained in previous assays with the p3E1.2 derived vectors by Lobo *et al.* (1999). If the length of the intervening

sequence was reduced to 40 bp or less, the frequency of transposition began to decrease dramatically (FIG. 2).

Example 3: Interplasmid Transposition Assay of pCRII-ITR and pBSII-ITR Plasmids

[61] According to an embodiment of the present invention, the excision assay described herein shows that a minimum of 163 bp of the 3' terminal region and 125 bp of the 5' terminal region (from the restriction site *Sac*I to the end of the element) may be used for excision, while the pIAO-P/L constructs showed that a minimal distance of 55 bp between termini may be utilized to effect movement. These data suggested that the inclusion of intact left and right terminal and internal repeats and spacer domains would be sufficient for transposition.

[62] The pCRII-ITR plasmid was constructed following PCR of the terminal domains from pIAO-P/L-589 using a single IR specific primer. A second construct pCRII- JFO3/04 was also prepared using two primers that annealed to the *piggyBac* 5' and 3' internal domains respectively, in case repeat proximate sequences were required.

[63] The interplasmid transposition assay was performed in *T.ni* embryos and the plasmids were recovered using LB/Kan/Cam plates (Sambrook *et al.*, 1989) with the controls plated on LB/Amp plates. A total of 10 randomly picked colonies were sequenced, and all were confirmed as resulting from transposition events, having the characteristic tetranucleotide TTAA duplication at the insertion sites. These insertion sites in pGDV1 were among the same previously described (Lobo *et al.*, 1999 and Thibault *et al.*, 1999). The sequencing results also confirmed that all 10 transposition events retained the expected terminal domain configurations. The frequency of transposition events was estimated at 2×10^{-4}, a similar frequency to that obtained with non-mutagenized constructs for this species (Lobo *et al.*, 1999).

[64] Independent verification that the 702 bp PCR cloned fragment (ITR cartridge, FIG. 3(C1)) may be used as a cartridge to generate transpositionally competent plasmids was obtained by excising the *Bam*HI fragment from pCRII-ITR, and ligating it into the pBlueScript II (Stratagene) plasmid to construct pBSII-ITR. Frequencies similar to those for the pCRII-ITR construct in the interplasmid transposition assay, were obtained.

Example 4: Construction of Minimum *PiggyBac* Vector pXL-Bac

[65] A new *piggyBac* minimum vector pXL-Bac (FIG. 3(C2)) was also constructed by combining the 702 bp *Bam*HI ITR fragment with the pBlueScript II *Bam*HI fragment and inserting a PCR amplified pBSII multiple cloning site (MCS) between the terminal repeats. The pXL-Bac vector was tested by inserting an *Xba*I fragment from πKOα (obtained from A, Sarkar, University of Notre Dame), containing the Kanamycin resistance gene, *E. coli* replication origin, and *Lac* a-peptide, into the MCS of pXL-Bac to form pXL-Bac-KOa. Interplasmid transposition assays yielded a frequency of over 10^{-4} for transposition of the modified ITR sequence, a similar level as observed for the intact *piggyBac* element.

[66] ***Example 5***: Derivative Vectors of pXL-Bac

[67] Using the pXL-Bac minimal vector, several derivative vectors may be constructed containing marker genes for detection of successful transformations. In one example, the vectors pXL-Bac-EYFP, pXL-Bac-EGFP, and pXL-Bac-ECFP (FIGS. 15-17) were assembled to contain the 3XP3 promoter driven flourescent protein genes of Horn and Wimmer (2000) by PCR amplifying these sequences from their respective *piggyBac* vectors using the primers E*FP-for (5' ACGACTAGTGTTCCCACAATGGTTAATTCG 3') (SEQ ID NO: 2) and E*FP-rev (5' ACGACTAGTGCCGTACGCGTATCGATAAGC 3') (SEQ ID NO: 3) each terminating in an *Spe*I restriction endonuclease site, and inserting these fragments into the *Spe*I digested pXL-Bac vector at the unique *Spe*I site of the multiple cloning site. Vectors constructed in this fashion allow detection of successful transformation by the pXL-Bac vector and may be further modified to include a separate gene of choice and suitable promoter adjacent to the marker gene in the multiple cloning site.

Example 6: Derivative Vectors of pCRII-ITR or pBSII-ITR

[68] Similar modifications may be made to either the pCRII-ITR or the companion vector, pBSII-ITR, by inserting a marker gene into the plasmid adjacent to the ITR cartridge of these plasmids. In one example, the plasmids pBSII-ITR-ECFP, pBSII-ITR-EGFP, and pBSII-ITR-EYFP (FIGS. 18-20) were constructed using the strategy described in Example 5 to PRC amplify an *Spe*I fragment containing the marker genes from the Horn and Wimmer (2000) *piggyBac* vectors and insert them into the unique *Spe*I site of the pBSII-ITR plasmid.

Example 7: Facilitating Expression of the Transposase

[69] Expression of the transposase is important in gaining movement of any of the vectors described herein. To facilitate expression of the transposase, a *Bam*HI cartridge containing only the *piggyBac* open reading frame sequences was PCR amplified from the *piggyBac* transposon clone p3E1.2 using the primers BamH1E-for1 (5' GCTTGATAAGAAGAG 3') (SEQ ID NO: 4) and BamH1E-rev1 (5' GCATGTTGCTTGCTATT 3') (SEQ ID NO: 5). This cartridge was then cloned into the pCaSpeR-hs vector at a unique *Bam*H1 site downstream of the Drosophila heat shock promoter (pCaSpeR-hs-orf) to effect heat shock induced expression of the *piggyBac* transposase following co-injection with any *piggyBac* vector.

Example 8: In Vitro Expression of mRNA of *PiggyBac* Transposase

[70] In some eukaryotic systems, the heat shock promoter may not function to express the transposase protein. An additional plasmid was constructed to allow in vitro expression of the messenger RNA sequence of the *piggyBac* transposase. Co-injection of this mRNA into embryos along with the *piggyBac* vectors would allow translation of the *piggyBac* transposase without having to rely on the expression of the mRNA from a promoter which may or may not be active in the desired system. In addition, this strategy provides much more transposase protein in the embryos, leading to a greater mobility of the *piggyBac* vectors. The *Bam*HI cartridge was excised from the plasmid pCaSpeR-hs-orf by restriction digestion with *Bam*HI and ligated into a *Bam*HI digested commercially available vector; pBSII (Stratagene) to make pBSII-IFP2orf (FIG. 6), allowing in vitro transcription of the *piggyBac* transposase mRNA under control of the bacteriophage T7 promoter.

Example 9: Alternative Promoters for the *PiggyBac* Transposase Gene

[71] Further modification of pBSII-IFP2orf may be effected to introduce alternative promoters that would drive expression of the *piggyBac* transposase gene. Three examples are provided. pBSII-hs-orf (FIG. 7) was constructed by excising the heat shock promoter region from pCaSpeR-hs using *Eco*R I and *Eco*R V digestion followed by blunt end polishing of the *Eco*RI terminus, and ligating the fragment to the blunt end polished *Eco*RI/*Hin*dIII digested pBSII-IFP2orf plasmid. The plasmid pBSII-IE1-orf was prepared by PCR amplification of the IE1 promoter from the plasmid pIE1FB using the primers IE1-Ac-for (5' ACGTAAGCTTCGATGTCTTTGTGATGCGCC 3') (SEQ ID NO: 6) and IE1-Ac-rev (5' ACGGAATTCACTTGCAACTGAAACAATATCC 3') (SEQ ID NO: 7) to generate an

*Eco*RI/*Hin*dIII tailed fragment that was then inserted into *Eco*RI and *Hin*dIII digested pBSII-IFP2orf. This plasmid allows constitutive expression of the *piggyBac* transposase in a diversity of eukaryotic systems. A final demonstration was prepared by digesting the plasmid pHAct5cEGFP (Pinkerton *et al.*, 2000) with *Bam*HI and *Eco*RI to recover the Drosophila Actin 5c promoter which was then inserted into pBSII digested with *Eco*RI and *Bam*HI. The *Bam*HI cartridge from pCaSpeR-hs-orf was excised by digestion with *Bam*HI and cloned downstream of the Actin 5c promoter at the unique *Bam*HI to form the plasmid pBSII-Act5c-orf (FIG. 21). This allows high level expression of the *piggyBac* transposase in embryos of insects.

Example 10: Transposase Expression in Vertebrate Systems

[72] While all of the constructs in Example 9 permit expression of the transposase in insect systems, they may not permit optimal expression of the transposase in vertebrate systems. Using the commercially available pDsRed1-N1 plasmid (Clonetech) the *Bam*HI cartridge was cloned from pBSII-IFP2orf into the *Bam*HI site adjacent to the CMV promoter to effect efficient expression of the *piggyBac* transposase in vertebrate systems. This plasmid was further modified by adding the 3XP3 promoter through PCR amplification of this promoter from the plasmid pBacI[3XP3-EYFPafm] (Horn and Wimmer, 2000) using the primers 3XP3-for (5' ACTCTCGAGGTTCCCACAATGGTTAATTCG 3') (SEQ ID NO: 8) and 3XP3-rev (5' ACTGAATTCATGGTGGCGACCGGTGGATCG 3') (SEQ ID NO: 9) to generate a *Xho*I/*Eco*RI tailed cartridge that was then cloned into the *Xho*I and *Eco*RI digested pDsRed1-N1 backbone to generate the plasmid p3XP3-DsRed-orf (FIG. 9).

Example 11: Optimizing *PiggyBac*

[73] In some cases it may be preferable to inject transposase protein to permit movement of the *piggyBac* transposon. The natural *piggyBac* transposase sequence is not efficiently expressed in prokaryotic systems due to a preponderance of eukaryotic codons. To achieve better expression of the *piggyBac* transposase in bacterial systems for purification and functional utility a sequence called optimized *piggyBac* orf (FIG. 23) was created, substituting prokaryotic codon biases wherever possible. This sequence generated the same protein sequence, but represents an artificial gene expressing the *piggyBac* transposase.

MATERIALS AND METHODS FOR EXAMPLES 1-11

Plasmids

[74] p3E1.2 deletion series: The p3E1.2 plasmid (Fraser *et al.*, 1995) was first linearized using the restriction sites *Bam*HI and *Eco*RI, blunt ended with the klenow fragment, then religated to construct the p3E1.2(DMCS) eliminating the MCS of the pUC18 sequence. Internal deletions were made using the Erase-A-Base System (Promega). p3E1.2(DMCS) was cut at the unique *Sac*I site within the *piggyBac* element, generating an *Exo*III resistant end, and then cut at the *Bgl*II site to generate an *Exo*III sensitive end. Fractions of the *Exo*III deletion reaction from the *Bgl*II site toward the 3' terminus were stopped every 30 seconds, and were blunt ended by S1 nuclease, recircularized, and transformed into DH5a cells. Recovered plasmids were size analyzed using a quick screen method (Sekar, 1987). The presence of intact 3' termini was confirmed using a *Bsi*WI digestion, and then sequenced. Nine consecutive plasmids in the size range of approximately 100~200 bp deletions were recovered and named p3E1.2-d-1 to p3E1.2-d-9, with p3E1.2-d-9 having the maximum deletion (FIG. 1).

[75] pIAO-P/L series: The p3E1.2 B/X plasmid was constructed as a pCRII TA clone (Invitrogen) of the entire *piggyBac* transposon and flanking TTAA targets sites following PCR from the plasmid p3E1.2 using the *Bam*HI/*Xba*I-tailed primer M1F34 (5'-GGATCCTCTAGATTAACCCTAGAAAGATA-3') (SEQ ID NO: 10). The element and flanking TTAA sites were then excised using the enzyme *Bam*HI and ligated to form a circular molecule. Two outward facing internal *piggyBac* primers, one with a terminal *Apa*I site (5'-GAAAGGGCCCGTGATACGCCTATTTTTATAGGTT-3') (SEQ ID NO: 11) and the other with a terminal *Kpn*I site (5'-AATCGGTACCAACGCGCGGGGAGAGGCGGTTTGCG-3') (SEQ ID NO: 12), were used to generate a linear *ApaI*/*Kpn*I-tailed fragment. This fragment was ligated to a PCR fragment containing the beta-1 actamase gene and *E. coli* replication origin amplified from pUC18 using an *Apa*I-tailed primer (5'-CCAAGGGCCCTGACGTGAACCATTGTCACACGT-3') (SEQ ID NO: 13) and a *Kpn*I tailed (5'-TGTGGGTACCGTCGATCAAACAAACGCGAGATACCG-3) (SEQ ID NO: 14) primer pair. The resulting pIAO plasmid contains the circularized *piggyBac* transposon with ends separated by an 18 bp fragment of DNA having the restriction sites configuration *xbaI*/*Bam*HI/*xba*I, with a beta-lactamase gene and the *E. coli* origin of replication. The *lacZ* gene under the control of the polyhedron promoter was excised from pD-2/B-gal (Fraser *et*

al., 1996) using restriction enzymes *Nru*I and *Dra*I, and cloned into the unique *Hpa*I site within the *piggyBac* element of pIAO to form pIAO-polh/*lacZ* (pIAO-P/L) plasmid.

[76] The pIAO-P/L-TTAA1 plasmid was constructed by digesting pIAO-polh/*lacZ* with *Sph*I and *Bsi*WI, and the fragment containing the internal-*piggyBac* sequence was isolated. Two complementing oligonucleotides, *Sph*I (5'-CGTCAATTTTACGCAGACTATCTTTCTAGGG -3') (SEQ ID NO: 15) and TTAA-*Sph*I (5'- TTAACCCTAGAAAGATAGTCTGCGTAAAATTGACGCATG -3') (SEQ ID NO: 16), were annealed to form a *Sph*I site on one end and a TTAA overhang on the other end. A second pair of oligonucleotides, *Bsi*WI (5'-GTACGTCACAATATGATTATCTTTCTAGGG-3') (SEQ ID NO: 17) and TTAA-*Bsi*WI (5'-TTAACCCTAGAAAGATAATCATATTGTGAC-3') (SEQ ID NO: 18) were annealed to form a *Bsi*WI site on one end and a TTAA overhang on the other. These two primer pairs were joined using the TTAA overlaps and inserted into the *Sph*I and *Bsi*WI sites of the digested pIAO-polh/*lacZ* plasmid to form the circular pIAO-P/L- TTAA1 plasmid.

[77] The pIAO-P/L-TTAA2 plasmid was constructed in a similar manner by combining the *Sph*I-terminal primer with TTAATTAA-*Sph*I (5'-TTAATTAACCCTAGAAAGATAGTCTGCGTAAAATTGACGCATG-3') (SEQ ID NO: 19), and the *Bsi*WI primer with TTAATTAA-*Bsi*WI (5'-TTAATTAACCCTAGAAAGATAATCATATTGTGAC-3') (SEQ ID NO: 20).

[78] The plasmids pIAO-P/L-2.2kb, pIAO-P/L-589bp, pIAO-P/L-354bp, pIAO-P/L-212bp and pIAO-P/L-73bp were constructed by insertion of *Hin*dIII or *Pvu*II fragments from the bacteriaphage lambda into the blunt ended *Xba*I site between the adjacent TTAA target sites of pIAO-polh/*lacZ*.

[79] Plasmids pIAO-P/L-55bp, pIAO-P/L-40bp and pIAO-P/L-22bp were constructed by annealing oligonucleotide pIAO-4501 (5'-CTAGTACTAGTGCGCCGCGTACGTCTAGAGACGCGCAGTCTAGAAD-3') (SEQ ID NO: 21) and pIAO-4502 (5'-TTCTAGACTGCGCGTCTCTAGACGTACGCGGCGCACTAGTACTAGD-3') (SEQ ID NO: 22), forming two *Xba*I sites and one *Spe*I site, and ligating them into the blunt ended pIAO-P/L *Xba*I fragment to generate pIAO-P/L-55bp. The pIAO-P/L-40bp plasmid was constructed by cutting pIAO-P/L-55bp plasmid at the *Xba*I sites of the inserted fragment and

then religating. Cutting pIAO-P/L-40bp at the *Xba*I and *Spe*I sites, and religating formed the pIAO-P/L-22bp plasmid.

[80] The pIAO-P/L-18bp plasmid was constructed by PCR amplification of the pIAO-P/L plasmid using the pIAO-18bp primer (5'-GATGACCTGCAGTAGGAAGACGD3') (SEQ ID NO: 23) and the TR-18bp primer (5'-GACTCTAGACGTACGCGGAGCTTAACCCTAGAAAGATAD3') (SEQ ID NO: 24). The amplified fragment was cut with *Xba*I and *Pst*I, and ligated to the pIAO-P/L *Xba*I and *Pst*I cut fragment.

[81] **pCRII-ITR, pCRII-JF03/04 and pBS-ITR plasmids**: The oligonucleotide ITR (5'-GGATTCCATGCGTCAATTTTACGCAD-3') (SEQ ID NO: 25), having the *piggyBac* IR and a terminal *Bam*HI site, was used to PCR amplify the *piggyBac* 3' and 5' IRs and TRs along with their spacer regions from the pIAO-P/L-589bp plasmid. The PCR fragment was TA cloned into pCRII (Invitrogen). The resulting plasmid, pCRII-ITR, replaces the entire internal sequence of *piggyBac* with the pCRII plasmid sequences. A second plasmid, pCRII-JF03/04, was constructed using the same strategy with the primers JFO3 (5'-GGATCCTCGATATACAGACCGATAAAAACACATGD-3') (SEQ ID NO: 26) and JF04 (5'- GGTACCATTGCAAACAGCGACGGATTCGCGCTATD-3') (SEQ ID NO: 27). JFO3 is 83 bp internal to the 5' terminus, JF04 is 90 bp internal to the 3' terminus. To construct the pBS-ITR plasmid, the 702 bp *Bam*HI fragment was excised from the pCRII-ITR plasmid and inserted into the *Bam*HI site of the pBlueScript (Stratagene) plasmid.

[82] **pXL-Bac plasmid**: The 702 bp fragment containing the *piggyBac* terminal repeats isolated from pCRII-ITR plasmid *Bam*HI digestion was religated to form a circular molecule, followed by *Bss*HII digestion. The pBlueScript II plasmid was also digested by *Bss*HII and the large fragment was band isolated. These two fragments were ligated together to form the pBSII-ITR(Rev) plasmid. The Multiple Cloning Site(MCS) was PCR amplified from the pBSII plasmid using the MCS for (5'-ACGCGTAGATCTTAATACGACTCACTATAGGG-3') (SEQ ID NO: 28) and MCS-rev (5'-ACGCGTAGATCTAATTAACCCTCACTAAAGGG-3') (SEQ ID NO: 29) primers, and cloned into *Bam*HI site of pBSII-ITR(Rev) to construct the pXL-Bac plasmid.

[83] The pXL-Bac minimum *piggyBac* vector was constructed by circularizing an ITR *Bam*HI fragment, followed by *Bss*HII digestion. The resulting *Bss*HII fragment was then

ligated to the pBlueScript II *Bss*HII AMP/ori containing fragment. The multiple cloning site was PCR amplified from pBSII plasmid and inserted into *Bam*HI site to form the pXL-Bac vector. Any desired gene may be inserted into the MCS [the *Bss*HII fragment taken from pBSII (Stratagene)] to construct a *piggyBac* transposon.

[84] **Helper plasmid**: phspBac (formerly pBhsDSac, Handler *et al.*, 1998) is a transposase-providing helper plasmid that expresses the *piggyBac* ORF under the control of the *D. melanogaster hsp70* promoter.

[85] **Target plasmid**: pGDV1 is a *Bacillus subtilis* plasmid (Sarkar *et al.*, 1997a) containing a chloramphenicol resistance gene, and is incapable of replication in *E. coli* unless provided with an *E. coli* origin of replication.

[86] **Microinjection**: *T. ni* embryos were collected approximately 2 hours post oviposition and microinjected as described by Lobo *et al.*, (1999). After injection, the embryos were allowed to develop for one hour at room temperature, heat shocked at 37 °C for one hour, and allowed to recover at room temperature overnight. Plasmids were recovered using a modified Hirt (1967) extraction procedure.

[87] **Excision Assay**: The excision assay was performed as described by Thibault *et al.*, (1999). Precise excision events were confirmed by sequencing using a fluorescent labeled M13 reverse primer (Integrated DNA Technologies, *Inc.*).

[88] **Interplasmid Transposition Assay**: The interplasmid transposition assay was performed as described by Lobo *et al.* (1999) and Sarkar *et al.* (1997a). Plasmids isolated from the injected and heat-shocked embryos, as well as those passaged through *E. coli* only, were resuspended in 20μl of sterile distilled water and 3μl of the DNAs were then electropotated into 10μl of competent *E. coli* DH 10B cells (Gibco-BRL) (Elick *et al.*, 1996a). A 1.0-ml aliquot of SOC (2% w/v Bactotryptone, 0.5% w/v Bacto yeast extract, 8.5 mM NaCl, 2.5 mM Kcl, 10 mM MgC_2 20 mM glucose) was added to the electroporated cells, and the cells were allowed to recover at 37°C for 15 minutes. An aliquot (1%) of the transformed bacteria was plated on LB plates containing ampicilin (100 μg/ml) and X-Gal (5-bromo-4-chloro-3-indolyl-β3-D-galactosidase; 0.025 μg/ml), and the rest were plated on LB plates containing kanamycin (10 μg/ml), chloramphenicol (10 μg/ml) and X-Gal (0.025 μg/ml). Restriction analysis using *Hin*dIII and *Eco*RV and PCR using outward facing primers specific to *piggyBac* (JF01: 5'-CCTCGATATACAGACCGATAAAACACATG-3'

(SEQ ID NO: 30) and JF02: 5'-GCACGCCTCAGCCGAGCTCCAAGGGCGAC-3' (SEQ ID NO: 31)) enabled the preliminary identification of clones with putative interplasmid transposition events. The right insertion site of the clones was sequenced, with the Thermo Sequenase fluorescence-labeled primer sequencing kit (Amersham) and an ALF Express Automated Sequencer (Pharmacia Biotech), using the fluorescence-labeled JF02 primer, while the left insertion site was sequenced with the MF 11 reverse primer (5'-GGATCCCTCAAAATTTCTTCTAAAGTA-3') (SEQ ID NO: 32).

[89] To check for plasmid replication in the embryos, Hirt-extracted plasmid DNAs recovered from injected *D. melanogaster* embryos were digested with the restriction enzyme *Dpn*I (Geier and Modrich, 1979). *E. coli* cells were transformed with equal volumes of the digested and undigested plasmid DNAs and plated on LB plates containing kanamycin, chloramphenicol and X-Gal as above.

[90] The pIAO-P/L series transposition events were sequenced using the fluorescent labeled MF 11-reverse primer (5'-GGATCCCTCAAAATTTCTTCTAAAGTA-3') (SEQ ID NO: 33) and JF02 primer (5'-GCACGCCTCAGCCGAGCTCCAAGCGGCGAC-3') (SEQ ID NO: 34), and the pCRII-ITR and pBSII-ITR transposition events were sequenced using fluorescent labeled M13 reverse primer.

[91] **Automatic Thermocycle Sequencing:** Sequencing was performed using the Thermo Sequenase Fluorescent Labeled Primer Sequencing Kit (Amersham) and the ALF Express Automated Sequencer (Pharmacia Biotech), following standard protocols provided by the manufacturers.

[92] **Other Plasmids**: FIGS. 12, 13 and 14 present alternative plasmids that may be useful for gene transfer.

Example 12

[93] The present invention also provides ID sequences adjacent to the TRD of the *piggyBac* transposon that contribute to a high frequency of germline transformation in *D. melanogaster*. The present invention provides an analysis of a series of PCR synthesized deletion vectors constructed with the 3xP3-ECFP gene as a transformation marker (Horn and Wimmer, 2000). These vectors define ID sequences immediately adjacent to the 5' TRD and 3' TRD adjacent ID sequences that effect efficient germline transformation of *D.*

melanogaster. Using this information, the present invention provides a new ITR cartridge, called ITR1.1K, and verifies its utility in converting an existing plasmid into a mobilizable *piggyBac* vector that enables efficient germline transformation. The present invention also provides a transposon-based cloning vector, pXL-BacII, for insertion of sequences within a minimal *piggyBac* transposon and verifies its capabilities in germline transformations.

MATERIALS AND METHODS FOR EXAMPLE 12

Plasmids

[94] The pCaSpeR-hs-orf helper plasmid was constructed by PCR amplifying the *piggyBac* open reading frame using IFP2orf_For and IFP2orf_Rev primers, cloning into the pCRII vector (Invitrogen), excising using *Bam*H I, and inserting into the *Bam*H I site of the P element vector, pCaSpeR-hs (Thummel, *et al.*, 1992). A single clone with the correct orientation and sequence was identified and named pCaSpeR-hs-orf (FIG. 24).

[95] The p(PZ)-Bac-EYFP plasmid was constructed from the p(PZ) plasmid (Rubin and Spradling, 1983) by digesting with *Hin*d III and recircularizing the 7kb fragment containing *LacZ, hsp70* and Kan/ori sequences to form the p(PZ)-7kb plasmid. The ITR cartridge was excised from pBSII-ITR (Li *et al.*, 2001b) using *Not* I and *Sal* I and blunt end cloned into the *Hin*d III site of the p(PZ)-7kb plasmid. A 3xP3-EYFP marker gene was PCR amplified from pBac{3xP3-EYFPafm} (Horn and Wimmer, 2000), digested with *Spe* I, and inserted into the *Xba* I site to form p(PZ)-Bac-EYFP. It contains the *LacZ* gene, *Drosophila hsp70* promoter, Kanamycin resistance gene, ColE1 replication origin, 3xP3-EYFP marker and the *piggyBac* terminal repeats-only ITR cartridge (FIG. 24).

[96] The pBSII-3xP3-ECFP plasmid was constructed by PCR amplifying the 3xP3-ECFP marker gene from pBac{3xP3-ECFPafm} (Horn and Wimmer, 2000) using the primer pair ExFP_For and ExFP_Rev, then digesting the amplified fragment with *Spe* I, and cloning it into the *Xba* I site of pBlueScript II plasmid (Stratagene).

[97] The *piggyBac* synthetic internal deletion plasmids were constructed by PCR amplification from the pIAO-P/L-589bp plasmid (Li *et al.*, 2001b) using a series of primers. A total of 9 PCR products were generated using the combination of IFP2_R4 against all five IFP2_L primers and IFP2_L5 against all four IFP2_R primers. Two additional PCR products were also obtained using the IPF2_R-TR + IFP2_L and IFP2_R1 + IFP2_L primer pairs.

These PCR products were then cloned into the pCR II vector using the TOPO TA cloning kit (Invitrogen), excised using *Spe* I digestion, and cloned into the *Spe* I site of the pBSII-3xP3-ECFP plasmid to form the *piggyBac* internal deletion series (FIG. 25). The pBSII-ITR1.1K-ECFP plasmid (FIG. 24) was constructed by cloning the *Eco*R V/*Dra* I fragment from pIAO-P/L-589bp, which contained both *piggyBac* terminal repeats, into the *Eco*R V site of pBSII-3xP3-ECFP. The pXL-BacII-ECFP plasmid (FIG. 24) was constructed by PCR amplifying the ITR1.1k cartridge from pBSII-ITR1.1k-ECFP plasmid using MCS_For and MCS_Rev primers flanking by *Bgl* II site, cutting with *Bgl* II, religating and cutting again with *Bss*H II, then inserting into the *Bss*H II sites of the pBSII plasmid.

[98] A separate cloning strategy was used to construct pBS-pBac/DsRed. The 731 bp *Ase* I-blunted fragment from p3E1.2, including 99 bp of 3' *piggyBac* terminal sequence and adjacent NPV insertion site sequence, was ligated into a unique *Kpn* I-blunted site in pBS-KS (Stratagene). The resulting plasmid was digested with *Sac* I and blunted, then digested with *Pst* I, and ligated to a 173 bp *Hinc* II-*Nsi* I fragment from p3E1.2, including 38 bp of 5' *piggyBac* terminal sequence. The pBS-pBac minimal vector was marked with polyubiquitin-regulated DsRed1 digested from pB[PUbDsRed1] (Handler and Harrell, 2001a) and inserted into an *Eco*R I-*Hind* III deletion in the internal cloning site within the terminal sequences.

Transformation of *Drosophila melanogaster*

[99] The *D. melanogaster* w^{1118} white eye strain was used for all microinjections employing a modification of the standard procedure described by Rubin and Spradling (1982), in which the dechorionation step was eliminated. Equal concentrations (0.5 ug/ul) of each of the internal deletion plasmids, or the control plasmid pBac{3xP3-ECFPafm}, were injected along with an equal amount of the pCaSpeR-hs-orf helper plasmid into fresh fly embryos followed by a one hour heat shock at 37 °C and recovery overnight at room temperature. Emerging adults were individually mated with w^{1118} flies, and progeny larvae were screened using an Olympus SZX12 fluorescent dissecting microscope equipped with GFP (480nm excitation/510nm barrier), CFP (436nm excitation/480nm barrier), and YFP (500nm excitation/530 barrier) filter sets. Two positive adults from each of the vials were crossed with w^{1118} to establish germline transformed strains. The pBS-pBac/DsRed1 minimal vector was also injected and screened under HQ Texas Red® set no. 41004 (Handler and Harrell, 2001a).

Direct PCR Analysis

[100] Genomic DNAs from each of the transformed stains, the w^{1118} wild type strain, and a *piggyBac* positive strain M23.1 (Handler and Harrell, 1999) were prepared using a modified DNAzol procedure. About 60 flies from each strain were combined with 150 ul of DNAzol (Molecular Research Center, Inc.) in a 1.5 ml eppendorf tube. The flies were homogenized, an additional 450 ul of DNAzol was added, and the homogenates were incubated at room temperature for one hour. The DNAs were extracted twice with phenol:chloroform (1:1 ratio), and the aqueous fractions were transferred to new tubes for precipitation of the DNA with an equal volume of 2-propanol. The DNA pellets were washed with 70% ethanol, air dried, and 150 ul of dH_2O containing 10 ug of RNase A was added and resuspended.

[101] Two sets of direct PCRs were performed to identify the presence of *piggyBac* sequences in transformed fly genomes. Primers MF34 and IFP2_L were used to identify the presence of the *piggyBac* 3' terminal repeat, while MF34 and IFP2_R1 were used for identifying the *piggyBac* 5' terminal repeat. To exclude the possibility of recombination, a second PCR was also performed using the IFP2_R1 and IFP2_L primers to amplify the external stuffer fragment (Li *et al.*, 2001) between the terminal repeat regions.

Southern Hybridization Analysis

[102] Southern hybridization analysis was performed using a standard procedure with minor modifications (Ausubel *et al.* 1994). Approximately 8 ug of genomic DNA (isolated as above) from each of the transformed fly strains was digested with 40 units of *Hin*d III for four hours, followed by agarose gel electrophoresis at 60 Volts for 4 to 5 hours. The gel was then denatured, neutralized and transferred to nylon membranes, and baked at 80 °C for four hours. The membranes were pre-hybridized in the hybridization buffer overnight. A synthetic probe was prepared by nick translation (Invitrogen kit) using ^{32}P labeled dGTP against the pBSII-ITR1.1K-ECFP plasmid template. The purified probe was hybridized at 65 °C overnight followed by several washes, and the membranes were first exposed on phosphor screens (Kodak) overnight for scanning with a Storm phosphor Scanner (Molecular Dynamics System), and then exposed on X-ray film (Kodak).

Universal PCR and Inverse PCR Analysis

[103] The *piggyBac* insertion sites in the transformed fly strains were identified using either universal PCR (Beeman *et al.*, 1997) or inverse PCR techniques (Ochman *et al.*, 1988). For the universal PCR, the IFP2_L (3' TR) or IPR2_R1 (5' TR) primer was combined with one of 7 universal primers during the first round of PCR (94°C 1 minute, 40°C 1 minute, 72°C 2 minutes, 35 cycles). 2 ul of the reaction mixture from the first round of PCR was then used for a second round of PCR (94°C 1 minute, 50°C 1 minute, 72°C 2 minutes, 35 cycles) using IFP2_L1 (3' TR) or iPCR_R1 (5' TR) together with a T7 primer (nested on the universal primer).

[104] Inverse PCRs were performed by digesting 5 ug of the genomic DNAs from each of the transformed strains completely with *Hin*P1 I for the 3' end or *Taq* I for the 5' end, followed by purification using the Geneclean kit (Q-Biogene) and self-ligation in a 100 ul volume overnight. The self-ligated DNAs were precipitated and resuspended in 30 ul ddH_2O. A portion of them were then used for first round PCR (94 °C 1 minute, 40 °C 1 minute, 72 °C 2 minutes, 35 cycles) with primer pairs IFP2_R1 + MF14 for the 5' end and JF3 + IFP2_Lb for the 3' end. 2 ul of the first round PCR products were used as templates for the second round PCR (94 °C 1 minute, 50 °C 1 minute, 72 °C 2 minutes, 35 cycles) using primer pairs iPCR_R1 + iPCR_6 for the 5' end and iPCR_L1 + MF04 for the 3' end. The pBSII-ITR1.1k-ECFP strains were slightly different, the primer pair iPCR_L1 + IFP2_L-R were used for the 3' end in the second round PCR. All the PCR products were cloned into the pCRII vector (Invitrogen) and sequenced. The sequences were used to BLAST search the NCBI database to identify the locations of the insertions. MacVector 6.5.3 (Oxford Molecular Group) and ClustalX (Jeanmougin *et al.*, 1998) were used for sequence alignments.

RESULTS

Transformation Experiments with Synthetic Deletion Constructs:

[105] Each of the *piggyBac* synthetic internal deletion plasmids was formed by PCR amplifying from the pIAO-P/L-589 plasmid (Li *et al.*, 2001) by PCR amplifying across the facing terminal repeats and spacer with primers that recognize 5' or 3' sequences adjacent to the respective TRDs (FIG. 24). The fragments generated were cloned into a pBSII-3xP3-ECFP plasmid and sequenced.

[106] Each of the synthetic deletion series plasmids and the control plasmid, pBac{3xP3-ECFPafm}, were co-injected with the *hsp70*-regulated transposase helper into w^{1118} embryos, with surviving adults backcrossed, and G1 adult progeny screened for fluorescence. Positive transformants exhibited fluorescent eyes with CFP and GFP filter sets but not with the YFP filter set. Transformation frequencies from all injections are listed in Table 1, below.

Table 1. Transformation of *Drosophila melanogaster*

Plasmid	Embryos Injected	Embryos Hatched	Adults Mated	Adults Survied	Transformants Lines (G_0)	Transformation Frequency
p(PZ)-Bac-EYFP	2730	376	217	83	1	0.6%
pBSII-ECFP-R1/L5	990	240	83	70	6	8.9%
pBSII-ECFP-R2/L5	620	75	21	16	2	12.5%
pBSII-ECFP-R3/L5	650	127	29	20	3	15.0%
pBSII-ECFP-R4/L5	730	182	39	31	4	12.9%
pBSII-ECFP-R4/L4	670	169	44	28	3	10.7%
pBSII-ECFP-R4/L3	710	147	44	31	3	9.7%
pBSII-ECFP-R4/L2	850	191	55	46	5	10.8%
pBSII-ECFP-R4/L1	990	231	75	86	0	0%
pBSII-ITR1.1K-ECFP	530	128	43	84	5	13.9%
pBSII-ECFP-R-TR/L	610	169	62	71	0	0%
pBSII-ECFP-R1/L	840	247	81	69	0	0%
pBac{3xP3-ECFPafm}	650	104	45	69	4	12.9%
pXL-BacII-ECFP	1020	181	42	36	8	22.2%
pBSII-ITR1.1k-ECFP*	515	120	48	22	8	36.4%
pXL-BacII-ECFP*	533	199	115	88	22	25.0%

* The injections were done independently (Handler lab) using a 0.4:0.2 ug/ul vector/helper concentration ratio of DNA. The p(PZ)-Bac-EYFP plasmid yielded a low transformation frequency of 0.6% compared to the control plasmid, pBac{3xP3-ECFPafm}frequency of 12.9% (Table 1).

[107] Eight of the eleven synthetic ID deletion plasmids yielded positive transformants at an acceptable (not significantly different from control, $P<0.05$) frequency. The 5' ID deletion constructs pBSII-ECFP-R1/L5, pBSII-ECFP-R2/L5, pBSII-ECFP-R3/L5 and pBSII-ECFP-R4/L5 had variable deletions of the *piggyBac* 5' ID, retaining sequences from 66 bp (nucleotides 36~101 of the *piggyBac* sequence, GenBank Accession Number: AR307779) to 542 bp (36~567 of the *piggyBac* sequence). Each of these 5' ID deletions yielded ECFP positive germ line transformants at frequencies from 8.9% to 15.0% (Table 1) when paired with 1kb of the 3' ID sequence (nucleotides 1454~2409 of the *piggyBac* sequence). These results suggested that a minimal sequence of no more than 66 bp of the 5' ID may be necessary for efficient germline transposition.

[108] The R4 minimum 5' ID sequence primer was then used in combination with a series of 3' ID deletion primers to generate the constructs pBSII-ECFP-R4/L4, pBSII-ECFP-R4/L3,

pBSII-ECFP-R4/L2 and pBSII-ECFP-R4/L1. Of these four constructs, only pBSII-ECFP-R4/L1, which represented the greatest deletion of 3' ID sequence (2284~2409 of the *piggyBac* sequence), failed to yield transformants. Once again, frequencies for the positive transformant constructs were similar to the control (Table 1). It was therefore deduced that the minimal 3' ID sequence requirement for efficient germline transformation was between 125 bp (L1) and 378 bp (L2) of the 3' TRD adjacent ID sequence.

Construction of the ITR1.1k minimal sequence piggyBac cartridge:

[109] To construct a minimal sequence cartridge using the information gained from the synthetic deletion analysis, combinations of 5' and 3' minimal sequences were assembled and their transformation capabilities were tested. The pBSII-ECFP-R-TR/L construct is composed of a 35 bp 5' TRD lacking any 5' ID sequence, coupled to a fragment containing the 65 bp 3' TRD and 172bp of the adjacent 3' ID sequence. This combination did not yield any transformants, confirming the necessity for having 5' ID sequences in combination with 3' ID sequences for efficient transformation. Unexpectedly, addition of 101 bp of the 5' ID sequences to the 5' TRD sequences in the construct pBSII-ECFP-R1/L was not sufficient to recover transformation capacity when paired with the 172bp 3' ID sequences, even though the lower limit of essential 5' ID sequences had been suggested to be 66 bp using pBSII-ECFP-R1/L5 (Table 1). Increasing the 5' ID sequences to 276 bp in the pBSII-ITR1.1k-ECFP plasmid recovered the full transformation capability when paired with the 172 bp 3' ID sequence (Table 2). The minimal operational requirement for 5' ID sequences is therefore between 276 and 101 bp when coupled to a minimal 3' ID sequence of 172 bp.

[110] Two independent verifications of the pBSII-ITR1.1k-ECFP plasmid transforming capabilities were conducted for transformation of *D. melanogaster* . These transformation experiments resulted in calculated frequencies of 13.9% (FIG. 24) and 36% (Table 1). The discrepancy in frequencies may be attributed to differences in injection protocols between labs. Unless otherwise indicated, the transformation frequencies presented in Table 1 and FIG. 24 were obtained with injections of 0.6:0.6 ug/ul vector:helper concentration ratios. The increased efficiency of transformation for pBSII-ITR1.1k-ECFP observed in the second independent trial seems to be related to a decreased vector:helper concentration in *D. melanogaster*.

[111] Five recovered pBSII-ITR1.1k-ECFP transformed strains were used to perform genetic mapping to identify their chromosome locations. Several of the strains had insertions on the second and third chromosomes (including strain 1), while strain 3 had an insertion on the X chromosome. Strain 1 and strain 3 were chosen for further analyses.

Direct PCR Analysis of Integrations:

[112] Genomic DNAs from each of the transformed strains obtained with the synthetic deletion constructs in FIG. 24, as well as the *piggyBac* positive strain M23.1 and the negative white eye strain w^{1118}, were used to perform two sets of PCRs to verify the presence of the *piggyBac* 5' and 3' terminal repeat regions. An additional negative control PCR was performed on all transformants to show the absence of the external lambda phage DNA stuffer sequence (FIG. 26).

[113] The first set of PCRs utilized the IFP2_R1 and MF34 primers to amplify the 5' terminal repeat regions, and the second set of PCRs used the IFP2_L and MF34 primers to amplify the 3' terminal repeat regions. All of the synthetic deletion transformed strains, the M23.1 control strain, and the plasmid control yielded a strong PCR product of the correct size for each of the primer sets, confirming the presence of both of the *piggyBac* terminal repeat regions in all of the transformed strains. Interestingly, the white eye strain w^{1118} yielded a very weak product of the correct size with the 5' terminal repeat PCR amplification, but failed to generate a product with the 3' terminal specific primer set.

[114] A third set of PCRs was performed using the IFP2_R1 and IFP2_L primers in an attempt to amplify the external lambda phage DNA stuffer sequence which would be present if an insertion resulted from recombination of the entire plasmid sequence rather than transposition. The control product from this PCR reaction is a 925 bp fragment, and no such corresponding fragments were generated with any of the transformed strain genomic DNAs.

Southern Hybridization Analysis:

[115] Southern hybridization analysis was performed to verify the copy number and further confirm transposition of the *piggyBac* deletion plasmids into the *Drosophila* genome (FIG. 27). Genomic DNAs from two of the pBSII-ITR1.1k-ECFP strains (strain 1 and strain 3) and one of each of the other strains were digested with *Hind* III, with the pBSII-ITR1.1k-ECFP plasmid *Hind* III digest as a plasmid control. The *Hind* III digestion of all transformed strains

will generate four fragments if transpositional insertion has occurred: the pBSII plasmid backbone fragment (2960 bp), the 3xP3-ECFP marker fragment (1158 bp), the *piggyBac* 5' terminus fragment and the *piggyBac* 3' terminus fragment. Using the pBSII-ITR1.1k-ECFP plasmid as probe, all four fragments generated by the *Hin*d III digestion may be detected.

[116] The diagnostic 2960 bp pBSII backbone and 1158 bp ECFP marker fragments were present in all of the transformed strains examined. All of these strains also exhibited at least two additional bands corresponding to the *piggyBac* termini and adjacent sequences at the integration site (FIG. 27). These results confirmed that the observed frequencies were the result of transpositional integrations.

Analysis of Insertion Site Sequences:

[117] To further verify that *piggyBac*-mediated transposition of the synthetic deletion constructs occurred in these transformants, individual insertion sites were examined by isolating joining regions between the transposon and genomic sequences using either universal PCR or inverse PCR. Subsequent sequencing analysis of these joining regions demonstrated that all of the insertions occurred exclusively at single TTAA target sites that were duplicated upon insertion, and all insertion sites had adjacent sequences that were unrelated to the vector. The two pBSII-ITR1.1k-ECFP strains 1 and 3 have a single insertion on the third and X chromosome respectively.

DISCUSSION

[118] Transformation results from synthetic unidirectional deletion plasmids suggest that no more than 66 bp (nt 36~101 of the *piggyBac* sequence) of the *piggyBac* 5' ID sequence and 378 bp (nt 2031~2409 of the *piggyBac* sequence) of the *piggyBac* 3' ID sequence are necessary for efficient transformation when these deletions are paired with long (378 or 311 bp, respectively, or longer) ID sequences from the opposite end of the transposon. The transformation data from the pBSII-ITR1.1k-ECFP plasmid further defines the 3' ID essential sequence as 172 bp (nt 2237~2409 of the *piggyBac* sequence). Combining this same 172 bp 3' ID sequence with only the 5' TRD in the pBSII-ECFP-R-TR/L plasmid yielded no transformants, demonstrating that the 3' ID sequence alone was insufficient for full mobility. Unexpectedly, adding the 66 bp 5' ID sequence in pBSII-ECFP-R1/L also does not allow recovery of full transformation capability in spite of the fact that the same 66 bp does allow

full transformation capability when coupled to the larger (378 bp) 3' ID sequence in the pBSII-ECFP-R1/L2. This result cannot be explained by size alone, since the ITR cartridge strategy used to test this deletion sequence construct effectively replaces the rest of the *piggyBac* ID with the 2961 bp pBSII plasmid sequence.

[119] There appears to be an important sequence in the additional 206 bp of the L2 3' ID sequence that compensates for the smaller 5' ID sequence of R1. The data infer that an analogous sequence at the 5' end should be located within the 210 bp added to the 5' ID sequence in construction of the pBSII-ITR1.1k-ECFP, since this construct exhibits full transforming capability using the L 3' ID sequence. Aligning these two sequences using MacVector 6.5.3 identified two small segments of repeat sequences common between these approximately 200 bp sequences. These repeats, ACTTATT (nt 275~281, 2120~2126 and 2163~2169 of the *piggyBac* sequence) and CAAAAT (nt 185~190, 158~163 and 2200~2205 of the *piggyBac* sequence), occur in direct and opposite orientations, and are also found in several other locations of the *piggyBac* ID (FIG. 28). It seems that a minimum of one set of these repeats on either side of the internal domains are required for the transposon to permit full transforming capability.

[120] All documents, patents, journal articles and other materials cited in the present application are hereby incorporated by reference.

[121] Although the present invention has been fully described in conjunction with several embodiments thereof with reference to the accompanying drawings, it is to be understood that various changes and modifications may be apparent to those skilled in the art. Such changes and modifications are to be understood as included within the scope of the present invention as defined by the appended claims, unless they depart therefrom.

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